Method for preserving and/or storing cells which possess
nitrilase or nitrile hydratase activity

5 Description

The invention relates to a method for preserving and/or storing
microorganisms which exhibit at least one nitrile hydratase or
nitrilase enzyme activity, with the preservation and/or storage
10 being effected in an aqueous medium which comprises at least one
aldehyde, with the total aldehyde concentration being in a range
from 0.1 to 100 mM/l.

Enzymes which are produced by microorganisms are being used
15 increasingly as biocatalysts in chemical production processes. In
particular, the enzymic hydrolysis of nitriles to give amides,
carboxylic acids or α-hydroxycarboxylic acids is a process of
great economic importance. Nitrile-hydrolyzing enzymes can be
subdivided into the nitrile hydratase and nitrilase families. In
20 their active centers, nitrile hydratases and nitrilases possess a
cysteine molecule which is essential for the catalysis
(Levy-Schil (1995) Gene 161:15-20). The nitrile hydratases
catalyze the addition of one molar equivalent of water to give
the corresponding amides. Nitrilases catalyze the addition of two
25 molar equivalents of water to give the corresponding carboxylic
acids. As a rule, the said enzymes bring about an optically
selective hydration or hydrolysis, leading to optically active
(chiral) products. Chiral carboxylic acids are sought-after
compounds for synthetic organic chemistry. They are starting
30 compounds for a large number of pharmaceutical active compounds
or active compounds for plant protection. Chiral carboxylic acids
can be used for the classical racemate resolution by way of
diastereomeric salts. Thus, R-(-)- or S-(-)-mandelic acid is
used, for example, for the racemate resolution of racemic amines.
35 R-(-)-mandelic acid is also used as an intermediate in synthesis.

While purified or partially purified enzymes are usually employed
for the enzymic reactions, it is also possible to use
microorganisms which possess corresponding enzyme activities. The
40 enzymes can be of natural or recombinant origin. As a rule, the
enzymes are prepared (expressed) in a step which proceeds the
reaction. In this connection, it is desirable to prepare
relatively large quantities of enzyme and introduce these
quantities into the catalytic process as needed. However, this
45 makes it necessary to store the enzyme while retaining its
activity. Cooling and/or freezing are standard methods in this
connection. However, freezing usually requires complex

freezing/thawing methods and is as a rule associated with a serious loss of enzyme activity. In general, cooling is associated with elaborate logistics and with energy costs.

5 EP-A1 0 666 320 describes a method for preparing α-hydroxyacids/amides from the corresponding nitrile, with the microorganisms employed being incubated in the presence of sodium sulfite (1 M) and phosphate buffer (50 mM) prior to the reaction. Furthermore, the enzyme activity can be further stabilized during 10 the reaction by adding phosphite or hypophosphite, with said additions resulting in free, enzyme-inhibiting aldehyde being complexed. EP-A1 0 610 048 describes a microbial method for preparing α-hydroxyacids, with the enzyme activity being stabilized during the reaction by adding sodium sulfite, which 15 likewise results in free, enzyme-inhibiting aldehyde being complexed. In said methods, the additives are without exception added during the conversion of the nitrile. Methods for stabilizing prior to use in the reaction have not been disclosed.

20 The typical method for preserving cysteine-dependent activities is that of adding dithiothreitol and/or mercaptoethanol and/or ethylenediaminetetraacetic acid (example: Rhodococcus rhodochrous J1 nitrilase; Kobayashi M (1989) Eur J Biochem 182: 349-356).

25 The stabilization of Rhodococcus sp. ATCC 39484 nitrilase was achieved by adding substrate (benzonitrile) (Stevenson DE (1992) Biotechnol Appl Biochem 15:283-302). In the case of Rhodococcus rhodochrous NCIB 11216 nitrilase, a basic pH, the temperature and the enzyme concentration are responsible, in addition to the 30 substrate concentration, for the speed of the stabilization (Harper BH (1976) Biochem Soc Trans 4:502-504; Harper BH (1977) Biochem J 165:309-319). A disadvantage in these cases is that the stabilizer is transformed by the enzyme and consequently loses its effect over time.
35
The addition of inorganic salts (including up to 20% $(NH_4)_2SO_4$) and alcohols (up to 50% glycerol, 10% ethanol) for the purpose of stabilizing enzyme activity has been described in the case of Rhodococcus rhodochrous J1 nitrilase (Nagasawa T (2000) Eur J 40 Biochem 267:138-144).

The addition of 60% ammonium sulfate, 2M NaCl or 30% propanediol for the purpose of stabilizing enzyme activity has been described in the case of Alcaligenes faecalis JM3 nitrilase (Nagasawa T 45 (1990) Eur J Biochem 194:765-772).

EP-A1 0 707 061 describes methods for stabilizing nitrilase-comprising cells by adding inorganic salts (phosphates, borates, sulfates, sulfites and hydrochlorides), at concentrations of at least 100 mM up to the saturation limit, to
5 the storage buffer.

US 4,931,391, EP-A1 0 243 967 and US 4,900,672 describe the stabilization of a nitrile hydratase activity by adding amides or carboxylic acids (or a combination of substances) to the cell
10 suspension.

US 4,343,900 describes a method for producing acrylamide from acrylonitrile, with alkali metal carbonates being added to the reaction mixture for the purpose of avoiding the loss of activity
15 in connection with the swelling of the fixed cells which are used.

US 6,251,646 and US 6,368,804 describe methods for stabilizing nitrilase activity-harboring microorganisms by adding ammonium,
20 sodium or potassium (hydrogen) carbonates at concentrations of from at least 0.1 M up to the saturation concentration.

Because of the reactive aldehyde group, aldehydes are classified as being enzyme-inhibiting substances. Their inhibitory effect on
25 nitrilases during the production process is emphasized in a large number of publications (EP-B1 0 773 297 B1, p. 4 paragraphs [0013] and [0025]; EP-B1 0 707 061 B1, p. 2 paragraph [0005]; EP-B1 0 666 320, p. 2 paragraph [0004] and the literature references which are cited at that point; EP-A2 0 486 289 p. 2
30 line 30, and the literature references which are cited at that point; Yamamoto (1992) J Ferm Technol 73:425-430, in particular p. 429 last paragraph).

The inactivation of the nitrilase/nitrile hydratase activity
35 during storage is an important cost factor in connection with using said enzymes industrially. For example, at 4°C and pH 6.0, the activity decreases by 36% over a period of 6.6 days, denoting an activity loss of 5.5% per day (cf. Fig. 1; comparison experiments in Example 3). Loss of activity in the case of
40 nitrilases can be due, for example, to the enzyme multimer decomposing into its monomers, which do not possess any nitrilase activity (Nagasawa T (1990) Eur J Biochem 194:765-772). The described methods are only to a very limited extent able to solve this problem. Furthermore, said methods use high concentrations
45 of additives for stabilizing the biocatalysts, which additives

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have furthermore to be separated off, and disposed of, in an elaborate manner after the biocatalyst has been used.

5 The object underlying the present invention was consequently that of providing a method which enables a nitrilase/nitrile hydratase activity to be stabilized for as long as possible without the reaction mixture being contaminated with unwanted attendant substances.

10 The method according to the invention achieves this object.

A first step in the invention relates to a method for preserving and/or storing microorganisms which possess at least one nitrile hydratase or nitrilase enzyme activity, with the preservation 15 and/or storage being effected in an aqueous medium which comprises at least one aldehyde, with the total aldehyde concentration being in a range from 0.1 to 100 mM/l.

Said preservation step is preferably carried out before the cells 20 are treated with a reactant whose reaction is to be catalyzed by the cells. In a preferred embodiment, the aqueous medium comprises a total concentration of cyanide compounds, which are selected from the group consisting of nitriles, hydrocyanic acid and cyanide salts, which is at most 10 mol% of the total aldehyde 25 concentration. In a particularly preferred embodiment, the aqueous medium which is suitable for the preservation and/or storage does not comprise any additions of said cyanide compounds.
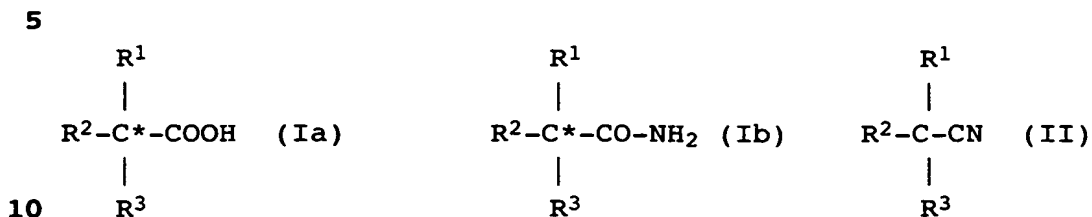
30 The term "aldehyde" is to be understood broadly and encompasses both aliphatic and aromatic aldehydes. In a preferred embodiment, aldehyde means compounds of the formula III:

$$R^6-C=O \quad (III)$$
35
$$|$$
$$H$$
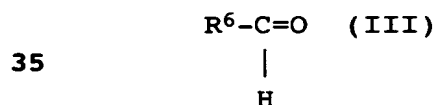
where R^6 can be substituted or unsubstituted, branched or unbranched, C1-C10-alkyl-, C2-C10-alkenyl-, or substituted or 40 unsubstituted aryl- or hetaryl-. Particular preference is given to aromatic aldehydes, with very particular preference being given to unsubstituted benzaldehyde and substituted benzaldehydes, such as o-chlorobenzaldehyde, m-chlorobenzaldehyde, p-chlorobenzaldehyde, o-bromobenzaldehyde, 45 m-bromobenzaldehyde, p-bromobenzaldehyde, o-methylbenzaldehyde, m-methylbenzaldehyde and p-methylbenzaldehyde.

5

The preserved/stored microorganisms can be used, for example, for converting racemic nitriles of the formula (II) into chiral carboxylic acids of the formula (Ia) or chiral amides of the formula (Ib):

5

$$
\begin{array}{ccc}
R^1 & R^1 & R^1 \\
| & | & | \\
R^2\text{--}C^*\text{--}COOH \quad (Ia) & R^2\text{--}C^*\text{--}CO\text{--}NH_2 \quad (Ib) & R^2\text{--}C\text{--}CN \quad (II) \\
| & | & | \\
R^3 & R^3 & R^3
\end{array}
$$

10

* an optically active center

15 R^1, R^2 and R^3 are, independently of each other, hydrogen, substituted or unsubstituted, branched or unbranched, C1-C10-alkyl-, C2-C10-alkenyl-, substituted or unsubstituted aryl-, hetaryl-, OR^4 or NR^4R^5, and where the radicals R^1, R^2 and R^3 are always different,

20 R^4 is hydrogen, substituted or unsubstituted, branched or unbranched, C1-C10-alkyl-, C2-C10-alkenyl-, C1-C10-alkylcarbonyl-, C2-C10-alkenylcarbonyl-, aryl-, arylcarbonyl-, hetaryl- or hetarylcarbonyl-,

25 R^5 is hydrogen, substituted or unsubstituted, branched or unbranched, C1-C10-alkyl-, C2-C10-alkenyl-, aryl- or hetaryl-.

Nitriles which are most preferred are mandelonitrile,
30 o-chloromandelonitrile, p-chloromandelonitrile, m-chloromandelonitrile, o-bromomandelonitrile, p-bromomandelonitrile, m-bromomandelonitrile, o-methylmandelonitrile, p-methylmandelonitrile or m-methylmandelonitrile. The most preferred chiral carboxylic
35 acids are R-mandelic acid, S-mandelic acid, R-p-chloromandelic acid, S-p-chloromandelic acid, R-m-chloromandelic acid, S-m-chloromandelic acid, R-o-chloromandelic acid, S-o-chloromandelic acid, S-o-bromomandelic acid, S-p-bromomandelic acid, S-m-bromomandelic acid,
40 S-o-methylmandelic acid, S-p-methylmandelic acid, S-m-methylmandelic acid, R-o-bromomandelic acid, R-p-bromomandelic acid, R-m-bromomandelic acid, R-o-methylmandelic acid, R-p-methylmandelic acid and R-m-methylmandelic acid.

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If α-hydroxy nitriles of the formula (IV)

$$R^6-\underset{\underset{OH}{|}}{C}-CN \quad (IV)$$
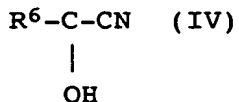
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(where the same definition as in formula (III) applies in the
case of R^6) are used as starting compounds for the sought-after
nitrilase/nitrile hydratase-catalyzed reaction, the aldehyde
employed for the preservation/storage is then preferably the same
10 aldehyde which yields said α-hydroxynitrile by reaction with
hydrocyanic acid or cyanide, i.e. the radical R^6 is preferably
chosen identically in the formulae III and IV.

The total concentration of aldehydes in the aqueous medium which
15 is suitable for the preservation and/or storage is from 0.1 to
100 mM/l, preferably from 0.2 to 50 mM/l, particularly preferably
from 0.5 to 10 mM/l, very particularly preferably from 0.3 to
5 mM/l, most preferably from 0.4 to 2 mM/l.

20 The aqueous medium can have a neutral, weakly basic or weakly
acidic pH. Accordingly, the pH is in a range from pH 6 to 8,
preferably pH 6.5 to 7.5. The preservation temperature is
preferably in a range from 0 to 40°C, particularly preferably from
1 to 10°C, very particularly preferably from 2 to 5°C.
25
The method according to the invention has proved, both under
laboratory conditions and under production conditions, to be
extremely suitable for ensuring long-lasting enzyme activity. The
biocatalyst does not exhibit any inactivation within the observed
30 period of 37 days.

Within the context of this invention, "microorganism" means
Gram-positive or Gram-negative bacteria.

35 Preference is given to all genera and species of the
Enterobacteriaceae, or families, and of the order
Actinomycetales, with very particular preference being given to
the Enterobacteriaceae species Escherichia, Serratia, Proteus,
Enterobacter, Klebsiella, Salmonella, Shigella, Edwardsielle,
40 Citrobacter, Morganella, Providencia and Yersinia.

Preference is furthermore given to the species Pseudomonas,
Burkholderia, Nocardia, Acetobacter, Gluconobacter,
Corynebacterium, Brevibacterium, Bacillus, Clostridium,
45 Cyanobacter, Staphylococcus, Aerobacter, Alcaligenes, Rhodococcus
and Penicillium.

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Most preference is given to Escherichia species, in particular Escherichia coli.

During the method according to the invention, the microorganism
5 can be present in a growing, resting, immobilized or disrupted state. "Disrupted cells" are to be understood, for example, as being cells which have been made permeable by a treatment with solvents, for example, or cells which have been broken open by means of an enzyme treatment, by means of mechanical treatment
10 (e.g. French press or ultrasonication) or by means of another method. The crude extracts which are obtained in this way are advantageously suitable for the method according to the invention. Partially or completely purified enzyme preparations can also be used for the method. Immobilized microorganisms or
15 enzymes, which can advantageously be used in reaction, are likewise suitable. The immobilization can be effected, for example, by adding one or more acrylic monomers (for example acrylamide, acrylic acid, methacrylamide, methacrylic acid, N,N-dimethylacrylamide, N,N-diethylacrylamide,
20 dimethylaminopropyl acrylate, dimethylaminopropyl methacrylate, dimethylaminopropylacrylamide, dimethylaminopropylmethacrylamide, diethylaminopropylacrylamide or diethylaminopropylmethacrylamide) and also, where appropriate, one or more crosslinking agents (e.g. methylenebisacrylamide, methylenebismethacrylamide,
25 1,2-dihydroxyethylenebisacrylamide or bisacrylamidoacetic acid) to the cell or enzyme preparation and then carrying out free-radical polymerization (initiated by, for example, ammonium persulfate).

30 In order to prevent contamination with foreign bacteria or fungi, suitable active compounds having an antibacterial or fungicidal effect, or other salts, such as ethylenediaminetetraacetic acid, can be added, where appropriate, to the preservation/storage solution.
35

The microorganisms which are used in methods according to the invention can, prior to preservation/storage, be cultured in a medium which enables these organisms to grow. This medium can be a synthetic medium or a natural medium. Media known to the
40 skilled person are used depending on the organism. To enable the microorganisms to grow, the media employed comprise a carbon source, a nitrogen source, inorganic salts and, where appropriate, small quantities of vitamins and trace elements.

45 Examples of advantageous carbon sources are polyols, such as glycerol, sugars, such as mono-, di- or polysaccharides, such as glucose, fructose, mannose, xylose, galactose, ribose, sorbose,

ribulose, lactose, maltose, sucrose, raffinose, starch or
cellulose, complex sugar sources, such as melasse, sugar
phosphates, such as fructose-1,6-diphosphate, sugar alcohols,
such as mannitol, alcohols, such as methanol or ethanol,
5 carboxylic acids, such as citric acid, lactic acid or acetic
acid, fats, such as soybean oil or rapeseed oil, amino acids,
such as an amino acid mixture, for example Casamino acids (Difco)
or individual amino acids, such as glycine or aspartic acid, or
amino sugars; the latter can also be used simultaneously as the
10 nitrogen source. Particular preference is given to polyols, in
particular glycerol.

Advantageous nitrogen sources are organic or inorganic nitrogen
compounds or materials which comprise these compounds. Examples
15 are ammonium salts, such as NH_4Cl or $(NH_4)_2SO_4$, nitrates, urea, or
complex nitrogen sources such as corn steep liquor, beer yeast
autolysate, soyabean meal, wheat gluten, yeast extract, peptone,
meat extract, caseine hydrolysate, yeast or potato protein, which
can frequently also simultaneously be used as the nitrogen
20 source.

Examples of inorganic salts are the salts of calcium, magnesium,
sodium, cobalt, molybdenum, manganese, potassium, zinc, copper
and iron. Anions of these salts which may in particular be
25 mentioned are the chlorine, sulfate and phosphate ions. An
important factor for increasing productivity in the method
according to the invention is the control of the Fe^{2+-} or Fe^{3+}-ion
concentration in the production medium.

30 Where appropriate, other growth factors, such as vitamins or
growth promoters, such as biotin, 2-KLG, thiamine, folic acid,
nicotic acid, pantothenate or pyridoxine, amino acids, such as
alanine, cysteine, proline, aspartic acid, glutamine, serine,
phenylalanine, ornithine or valine, carboxylic acids, such as
35 citric acid, formic acid, pimelic acid or lactic acid, or
substances such as dithiothreitol, are added to the nutrient
medium.

The ratio in which said nutrients are mixed depends on the nature
40 of the fermentation and is specified in each individual case. The
medium components can all be introduced at the beginning of the
fermentation, after they have, if necessary, been sterilized
separately or sterilized jointly, or else be subsequently added
during fermentation, continuously or discontinuously, as
45 required.

The culture conditions are specified such that the organisms grow so as to achieve the best possible yield (to be determined, for example, by the total activity of the recombinant protein which is expressed). Preferred culture temperatures are from 15°C to
5 40°C. Temperatures of between 25°C and 37°C are particularly advantageous. The pH is preferably maintained in a range from 3 to 9. pH values of between 5 and 8 are particularly advantageous. In general, an incubation period of from a few hours to a few days, preferably of from 8 hours to 21 days, particularly
10 preferably of from 4 hours to 14 days, is sufficient.

The skilled person can, for example, obtain information with regard to advantageously optimizing media from the textbook Applied Microbiol Physiology, "A Practical Approach (Eds. PM
15 Rhodes, PF Stanbury, IRL-Press, 1997, pp. 53-73, ISBN 0 19 963577 3).

The aldehyde can be added, for the purpose of preservation/storage, prior to, during or after the culture of
20 the microorganisms. Thus, it is possible, for example, to achieve maximum preservation of the activity by adding the aldehyde to the fermentation mixture without any further separation of the microorganisms.

25 However, it is likewise possible to separate the microbial cells or microorganisms, which have been cultured in this way, by, for example, centrifuging them from the culture medium, optionally washing them once, or several times, with a suitable buffer (such as a borate buffer or phosphate buffer) and then, for the purpose
30 of storage/preservation, taking them up, or resuspending them, in the aqueous solution, which comprises at least one aldehyde. The concentration of the microorganisms in said aqueous solution comprising at least one aldehyde can be selected at will.

35 The microorganisms which are used within the context of the invention exhibit at least one nitrile hydratase and/or nitrilase activity.

In a general manner, "nitrile hydratase" activity means the
40 property of catalyzing the addition of one molar equivalent of water to a nitrile, thereby forming the corresponding amide:

$$R-CN + H_2O \rightarrow R-CO-NH_2$$
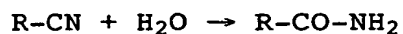
45 nitrile hydratases preferably comprise enzymes of the EC class 4.2.1.84 (nitrile hydratases).

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In a general manner, "nitrilase" activity means the property of catalyzing the addition of two molar equivalents of water to a nitrile, thereby forming the corresponding carboxylic acid:

5   $R-CN + 2\ H_2O \rightarrow R-COOH + NH_3$
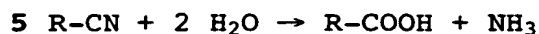
Nitrilases preferably comprise enzymes of the EC classes 3.5.5.1 (nitrilases), 3.5.5.2 (ricinin nitrilase), 3.5.5.4 (cyanoalanine nitrilases), 3.5.5.5 (arylacetonitrilases), 3.5.5.6 (bromoxynil 10 nitrilases) and 3.5.5.7 (aliphatic nitrilases).

The nitrilase and/or nitrile hydratase activity of said microorganism cells can be of natural or recombinant origin.

15 In this connection, "of natural origin" means that the microorganism as such, without any genetic change brought about by human action, exhibits a corresponding nitrilase and/or nitrile hydratase activity. A large number of such microorganisms are known to the skilled person. Preference is given, in 20 particular, to microorganisms of the genera Rhodococcus and Gordona, such as Rhodococcus sp. HT40-6 (FERM BP-5231), Rhodococcus rhodochrous ATCC 33278, Rhodococcus rhodochrous J-1 (FERM BP-1478) and Gordona terrae MA-1 (FERM BP-4535) (JP-A-4-222591, JP-B-6-55148, EP-A1 0 707 061).
25

In this connection, "of recombinant origin" means that the DNA sequence encoding an enzyme possessing nitrilase and/or nitrile hydratase activity is isolated from a microorganism and expressed in a microorganism of another species. Numerous sequences 30 encoding enzymes possessing nitrilase and/or nitrile hydratase activity are known to the skilled person. The following may be mentioned by way of example but not in a limiting manner:

    1.   Acidovorax facilis nitrilase 72W (Gavagan JE et al. (1999)
35   Appl Microbiol Biotechnol 52:654-659)
    2.   Acinetobacter sp. AK 226 nitrilase (Yamamoto K and Komatsu
          K (1991) Agric Biol Chem 55(6):1459-1466)
    3.   Acinetobacter sp. RFB1 nitrilase (Finnegan I et al. (1991)
          Appl Microbiol Biotechnol 36:142-144)
40 4.   Alcaligenes faecalis ATCC 8750 nitrilase (Yamamoto K et al.
          (1991) Appl Environ Microbiol 57(10):3028-3032)
    5.   Alcaligenes faecalis JM3 nitrilase (Nagasawa T et al.
          (1990) Eur J Biochem 194:765-772)
    6.   Arabidopsis thaliana nitrilases (NIT1/NIT2/NIT3) (Vorwerk S
45   et al. (2001) Planta 212:508-516
    7.   Arthrobacter sp. J-1 nitrilase (Bandyopadhyay AK et al.
          (1986) Appl Environ Microbiol 51(2):302-306)

8. Bacillus pallidus Dac521 nitrilase (Cramp R et al. (1997) Microbiology 143:2313-2320)

9. Comamonas sp. NI1 nitrilase (Cerbelaud E et al. (1996) Ind Chem Libr 8:189-200)

10. Comamonas testosteroni sp. nitrilase (Levy-Schil S et al. (1995) Gene 161:15-20)

11. Fusarium oxysporum f. sp. melonis nitrilase (Goldlust A and Bohak Z (1989) Biotechnol Appl Biochem 11:581-601)

12. Fusarium solani nitrilase (Harper BH (1977) Biochem J 167:685-692)

13. Klebsiella ozaenae nitrilase (McBride KE et al. (1986) Appl Environ Microbiol 52(2):325-330)

14. Pseudomonas fluoreszenz DSM 7155 nitrilase (Layh N et al. (1998) J Mol Catal B: Enzym 5:467-474)

15. Pseudomonas sp. nitrilase (Layh N et al. (1992) Arch Microbiol 158:405-411)

16. Pseudomonas sp. (S1) nitrilase (Dhillon J et al. (1999) Can J Microbiol 45: 811-815)

17. Pseudomonas sp. 13 nitrilase (Yanase H et al. (1982) Agric Biol Chem 46:2925)

18. Rhodococcus rhodochrous J1 nitrilase (Kobayashi M et al. (1989) Eur J Biochem 182:349-356)

19. Rhodococcus rhodochrous K22 nitrilase (Kobayashi M et al. (1990) J Bacteriol 172(9):4807-4815)

20. Rhodococcus rhodochrous NCIB 11215 nitrilase (Harper BH (1985) Int J Biochem 17(6):677-683)

21. Rhodococcus rhodochrous NCIB 11216 nitrilase (Harper BH (1977) Biochem J 165:309-319)

22. Rhodococcus rhodochrous PA34 nitrilase (Bhalla TC et al. (1992) Appl Microbiol Biotechnol 37:184-190)

23. Rhodococcus sp. ATCC 39484 nitrilase (Stevenson DE et al. (1992) Biotechnol Appl Biochem 15:283-302)

In a preferred embodiment, the nitrilase is described by an amino acid sequence which is encoded by a nucleic acid sequence which is selected from the group

a) a nucleic acid sequence having the sequence depicted in SEQ ID NO: 1,

b) nucleic acid sequences which are derived from the nucleic acid sequence depicted in SEQ ID NO: 1 as a result of the degeneracy of the genetic code,

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 1 which encode polypeptides having the amino acid sequences depicted in SEQ ID NO: 2 and exhibit at least 35%

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homology at the amino acid level and are able to convert at
least one nitrile into the corresponding carboxylic acid.

The expression of recombinant nitrilases/nitrile hydratases can
5 be effected, for example, using a suitable DNA construct which
has been introduced into the microorganism. The DNA construct is
preferably a vector. Vectors can, by way of example, be plasmids,
cosmids or phages. Preference is given to the vector being a
circular plasmid which comprises the nucleic acid sequence being
10 expressed in recombinant form and which is capable of autonomous
replication in the prokaryotic host cell. Vectors which may be
mentioned by way of example are:

a)   preferably pQE70, pQE60 and pQE-9 (QIAGEN, Inc.); pBluescript
15   vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A
      (Stratagene Cloning Systems, Inc.); ptrc99a, pKK223-3,
      pKK233-3, pDR540, pRIT5 (Pharmacia Biotech, Inc.); pLG338,
      pACYC184, pBR322, pUC18, pUC19, pKC30, pRep4, pHS1, pHS2,
      pPLc236, pMBL24, pLG200, pUR290, pIN-III^{113}-B1, λgt11 or pBdCI
20   in E.coli,

b)   preferably pIJ101, pIJ364, pIJ702 or pIJ361 in Streptomyces,

c)   preferably pUB110, pC194 or pBD214 in Bacillus,
25
d)   pSA77 or pAJ667 in Corynebacterium,

or derivatives of the abovementioned plasmids. Said plasmids
constitute a small selection of the possible plasmids. Other
30 plasmids are well known to the skilled person and are listed, for
example, in the book Cloning Vectors (Eds. Pouwels P. H. et al.
Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018).

The DNA construct comprises at least one nucleic acid sequence
35 which is to be expressed, which encodes an enzyme having
nitrilase and/or nitrile hydratase activity and which is
functionally linked to a promoter which functions in the
particular microorganism which is being used.

40 A large number of promoters which function in microorganisms are
known to the skilled person: promoters such as the cos, tac, trp,
tet, lpp, lac, lacIq, T7, T5, T3, gal, trc, ara, rha, SP6, λ-PR or
λ-PL promoters may be mentioned by way of example. Particular
preference is given to the E.coli rhamnose operon promoter (rha
45 promoter), which can be induced by adding rhamnose.

In a general manner, a functional linkage is understood as being an arrangement in which a genetic control sequence (e.g. a promoter) is able to exert its function in relation to the nucleic acid sequence which is to be expressed. In this
5 connection, function can, for example, denote control of the expression, i.e. transcription and/or translation, of the nucleic acid sequence. In this connection, control comprises, for example, the initiation, increase, regulation or suppression of the expression, i.e. transcription and, where appropriate,
10 translation. A functional linkage is understood, for example, as being the sequential arrangement of a promoter, of the nucleic acid sequence to be expressed and, where appropriate, of other regulatory elements, such as a terminator, such that each of the regulatory elements is able to fulfill its function in connection
15 with the expression of the nucleic acid sequence. The skilled person is familiar with a variety of ways for obtaining one of the DNA constructs according to the invention. The DNA construct can be prepared using customary recombination and cloning techniques, as are described, for example, in T Maniatis, EF
20 Fritsch and J Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in TJ Silhavy, ML Berman and LW Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, FM et al., Current Protocols in Molecular
25 Biology, Greene Publishing Assoc. and Wiley Interscience (1987).

Said DNA construct can comprise additional functional elements. The term functional elements is to be understood broadly and means all those sequences which exert an effect on the genesis,
30 the replication or the function of the DNA constructs or organisms according to the invention. For example, functional elements ensure, augment, regulate or modify transcription and, where appropriate, translation in corresponding host organisms.

35 Functional elements are described, for example, in "Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990)" or "Gruber and Crosby, in: Methods in Plant Molecular Biology and Biotechnology, CRC Press, Boca Raton, Florida, eds.: Glick and Thompson, Chapter 7, 89-108", as well as
40 in the citations which are contained in these publications. Different control sequences are suitable depending on the host organism or starting organism which is described in more detail below and which is converted into a genetically altered or transgenic organism as a result of introducing the expression
45 cassettes or vectors.

"Genetic control sequences" comprise, for example, the
5'-untranslated region or the noncoding 3' region of genes. In
addition, the term "genetic control sequences" means sequences
which encode fusion proteins which consist of a signal peptide
5   sequence. The following may be mentioned by way of example but in
a nonlimiting manner:

a)   Selection markers
     Selection markers are as a rule required for successfully
10   selecting transformed cells and for preventing the loss of
     the DNA construct from the host cell over time and during
     cell divisions. Such a loss can, in particular, occur if the
     recombinant protein which is encoded by the nucleic acid
     sequence to be expressed has a toxic effect on the
15   prokaryotic organism. The selectable marker which is
     introduced together with the expression construct confers
     resistance to a biocide (for example an antibiotic such as
     ampicillin, kanamycin or hygromycin) on the cells which have
     been successfully transformed. Selection markers which may be
20   mentioned by way of example are:

     - Amp (ampicillin resistance; b-lactamase)
     - Cab (carbenicillin resistance)
     - Cam (chloramphenicol resistance)
25   - Kan (kanamycin resistance)
     - Rif (rifampicin resistance)
     - Tet (tetracycline resistance)
     - Zeo (zeocin resistance)
     - Spec (spectinomycin)
30
     The selection pressure is maintained by using appropriate
     quantities of the antibiotic. The following may be mentioned
     by way of example: ampicillin, 100 mg/l, carbenicillin,
     100 mg/l, chloramphenicol, 35 mg/l, kanamycin, 30 mg/l,
35   rifampicin, 200 mg/l, tetracycline, 12.5 mg/l and
     spectinomycin, 50 mg/l.

     Selection markers furthermore comprise genes and gene
     products which, by, for example, complementing a genetic
40   deficiency in the amino acid or nucleotide synthesis, enable
     a correspondingly transformed host cell to be selected. Media
     which do not comprise said amino acid or said nucleotide
     building block are used, inter alia, for this purpose. The
     skilled person is familiar with a variety of such systems.
45   The deficiencies in tryptophan (e.g. trpC), leucine (e.g.
     leuB) and histidine (e.g. hisB) biosynthesis, as are present,
     for example, in the E.coli strain KC8 (Clontech), may be

mentioned by way of example. These deficiencies can be complemented, inter alia, by the selectable markers TRP1, Leu2 and HIS3.

5   b)   Transcription terminators
        The transcription terminator reduces an unwanted transcription and increases plasmid and mRNA stability.

    c)   Shine-Dalgarno sequences
10   A Shine-Dalgarno (SD) sequence is required for initiating translation and is complementary to the 3' end of 16S ribosomal RNA. The efficiency of the initiation of translation at the start codon depends on the actual sequence. An example of a consensus sequence suitable for
15   E.coli is: 5'-TAAGGAGG-3'. It is located approx. 4 to 14 nucleotides upstream of the start codon, with the optimum being 8 nucleotides. In order to avoid the formation of secondary structures (which can reduce expression), this region should preferably be rich in A/T nucleotides.
20
    d)   Start codon
        The start codon is the point at which translation is initiated. ATG is the start codon which is used the most in E. coli; GTG can also be used as an alternative.
25
    e)   Tags and fusion proteins

        N- or C-terminal fusions of the recombinant proteins to be expressed with relatively short peptides (tags) or other
30   proteins (fusion partners) may be advantageous. They can, for example, make it possible to achieve improvements in expression, solubility, detectability and purification. Preference is given to combining such fusions with protease cleavage sequences (e.g. for thrombin or factor X) which make
35   it possible to remove the tags or the fusion partner following expression and purification.

    f)   Multiple cloning regions (multiple cloning site; MCS) permit and facilitate the insertion of one or more nucleic acid
40   sequences.

    g)   Stop codon/translation terminators
        Of the three possible stop codons, preference is given to TAA since read-through, without any termination of the
45   translation, may possibly occur when TAG and TGA are used. A

sequence of several stop codons may also be used in order to
ensure reliable termination.

h)   Reporter genes
5   Reporter genes encode readily quantifiable proteins which, by
     way of intrinsic color or enzyme activity, make it possible
     to assess the efficiency of transformation, the level of
     expression and the site or time of expression. Reporter genes
     can, for example, encode the following proteins: hydrolases,
10   fluorescent proteins, bioluminescent proteins, glucosidases
     or peroxidases. Preference is given to luciferases,
     β-galactosidases, β-glucuronidase, green fluorescence
     protein, acetyl transferases, phosphotransferases and
     adenyltransferases (see also Schenborn E, Groskreutz D (1999)
15   Mol Biotechnol 13(1):29-44).

The preparation of a transformed microorganisms requires the
corresponding DNA (for example one of the expression cassettes or
vectors according to the invention) to be introduced into the
20 corresponding host cell. A large number of methods are available
for this procedure, which is termed transformation (see also
Keown et al. (1990) Methods in Enzymology 185:527-537). Thus, the
DNA can, by way of example, be introduced directly by means of
microinjection or electroporation or by means of bombarding with
25 DNA-coded microparticles (biolistic method using a
particle-bombardment gene cannon). It is also possible to
permeabilize the cell chemically, for example using polyethylene
glycol, such that the DNA is able to gain entry into the cell by
means of diffusion. The DNA [lacuna] can also be effected by
30 means of fusion with other DNA-containing units such as
minicells, cells, lysosomes or liposomes. Electroporation, in
which the cells are permeabilized reversibly by means of an
electric impulse, is another suitable method for inserting DNA.
Preferred general methods which may be mentioned are calcium
35 phosphate-mediated transformation, DEAE dextran-mediated
transformation, cationic lipid-mediated transformation,
electroporation, transduction and infection. These methods are
familiar to the skilled person and are, for example, described
(Davis et al.(1986) Basic Methods In Molecular Biology; Sambrook
40 J et al. (1989) Molecular cloning: A laboratory manual, Cold
Spring Harbor Laboratory Press; Ausubel FM et al. (1994) Current
protocols in molecular biology, John Wiley and Sons; Glover DM et
al. (1995) DNA Cloning Vol.1, IRL Press ISBN 019-963476-9).

45

Transformed cells, that is those which comprise the inserted DNA, can be selected from untransformed cells if a selectable marker forms part of the inserted DNA. A variety of selection markers are described above.

5

The invention furthermore relates to a preparation of microorganisms which contain at least one nitrile hydratase or a nitrilase enzyme activity, with the preparation comprising

10 a) at least one aldehyde giving a total aldehyde concentration in a range from 0.1 to 100 mM/l, and

b) cyanide compounds, selected in the group consisting of nitriles, hydrocyanic acid and cyanide salts, at a total
15 concentration which it at most 10 mol% of the total aldehyde concentration.

In a particularly preferred embodiment, the preparation according to the invention does not contain any additions of said cyanide
20 compounds.

The invention furthermore relates to the use of the preparation of microorganisms according to the invention for producing foodstuffs, feedstuffs, pharmaceuticals or fine chemicals. "Fine
25 chemicals" preferably means proteins, enzymes, vitamins, amino acids, sugars, fatty acids and natural and synthetic flavoring agents, aromatizing agents and dyes.

The invention furthermore relates to methods for preparing
30 recombinant proteins, enzymes (preferably enzymes possessing nitrilase and/or nitrile hydratase activity) or other fine chemicals such as amides or carboxylic acids (preferably chiral carboxylic acids and amides) using one of the preparation of microorganisms according to the invention or a preparation
35 thereof.

A preferred part of the subject-matter of the invention relates to a method for preparing carboxylic acids and/or amides (preferably chiral carboxylic acids/amides), comprising the
40 following steps:

a) culturing a microorganism which possesses at least one nitrile hydratase or nitrilase enzyme activity,

45 b) adding at least one aldehyde, with the total aldehyde concentration being in the range from 0.1 to 100 mM/l,

c)   bringing the aldehyde-treated preparation of said
      microorganisms into contact with at least one nitrile and
      converting said nitrile into a carboxylic acid and/or an
      amide.

5

In a preferred embodiment, the preparation of the microorganism
comprises, in connection with the addition of the aldehyde,
cyanide compounds, selected from the group consisting of
nitriles, hydrocyanic acid and cyanide salts, at a concentration
10   which is at most 10 mol% of the total aldehyde concentration. In
a particularly preferred embodiment, said preparation does not
contain any additions of said cyanide compounds. In an embodiment
which is furthermore preferred, the preparation can, after the
addition of the aldehyde (step b), be stored until being used in
15   reaction step c). The method according to the invention can be
carried out continuously or discontinuously in batch mode or
fed-batch mode. In this connection, both the preparation of the
microorganisms and the racemic nitrile, as substrate, can be
added subsequently.

20

Details with regard to carrying out the reactions and/or with
regard to purifying the products, etc., are described in detail,
for example, in WO 00/23577. The starting compounds, products and
methological parameters described in that publication are hereby
25   expressly incorporated by reference.

In another preferred embodiment, the method can be combined with
other methods for stabilizing, preserving and/or storing enzymes,
in particular nitrilases and/or nitrile hydratases. These methods
30   can, by way of example but not in a limiting manner, comprise:

a)   Adding at least one inorganic salt (preferably selected from
      the group consisting of phosphates, borates, sulfates,
      sulfites and hydrochlorides) at a concentration of at least
35   100 mM, preferably from 300 to 700 mM.

b)   Adding metal salts whose metal cation functions as a
      nitrilase and/or nitrile hydratase prosthetic group (e.g.
      cobalt chloride or iron sulfate).

40

c)   Adding nitriles (e.g. benzonitrile, isobutyronitrile or
      succinonitrile) and/or amides (s-caprolactam, isobutylamide
      or propionamide).

45 Figures

Fig.1:   Storage stability of a nitrilase for producing
        (R)-mandelic acid.

5   The figure depicts, by way of example, the decrease in
        the activity (A; in % of the initial activity) of three
        independent preparations of an E.coli-expressed nitrilase
        without added aldehyde (comparison experiments) over a
        period of up to 20 days (d).

10 Fig.2:   Storage stability of a nitrilase for producing
        (R)-mandelic acid.

        The figure depicts the decrease in the activity (A; in %
        of the initial activity) of preparations of an
15   E.coli-expressed nitrilase without added
        2-chlorobenzaldehyde (open circles) as compared with an
        otherwise identical preparation containing added
        2-chlorobenzaldehyde (closed circles). The figure depicts
        a period (t) of up to 32 days (d).

20
Examples

Unless otherwise described, general nucleic acid methods, such as
cloning, restriction cleavages, agarose gel electrophoreses,
25 linking of DNA fragments, transformation of microorganisms,
growth of bacteria and analysis of recombinant DNA sequences,
were carried out as described in Sambrook et al. (1989) (Cold
Spring Harbor Laboratory Press: ISBN 0-87969-309-6). Recombinant
DNA molecules were sequenced by the Sanger method (Sanger et al.
30 (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467) using an ABI
laser-fluorescence DNA sequencer. In order to avoid polymerase
errors in constructs to be expressed, fragments resulting from a
polymerase chain reaction were sequenced and checked.

35 Example 1: Preparing cells possessing nitrilase activity:

The *Escherichia coli* strain (TG10 pDHE1650 pAgro4 pHSG575) was
fermented in a 20 l bioreactor. The reactor, containing a 10 l
working volume, was inoculated with 200 ml of preliminary culture
40 from shaker flasks. The preliminary culture medium corresponds to
the main culture medium.

Medium:
    40 g   of 99.5% glycerol
45 15 g   of tryptone
    13.3 g   of potassium dihydrogen phosphate
    5 g   of yeast extract

20

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4 g          of diammonium hydrogen phosphate
1.7g         of citric acid
1.1 g        of magnesium sulfate heptahydrate
1 ml         of SL Korz 1000 C trace element solution
5 0.1 ml     of Tego KS 911 antifoaming agent
0.062 g      of iron(II) sulfate heptahydrate
10 mg        of thiamine hydrochloride
to 1 l       deionized water
```

10 The medium is sterilized at 121°C for 30 min. 0.1 g of ampicilin
is then added under sterile conditions.

```
Trace element solution
Citric acid*H20                                           20 g
15 Cobalt(II) chloride hexachloride     CoCl2 * 6H2O       2.5 g
Manganese(II) chloride tetrachloride MnCl2 * 4H2O          3.0 g
Copper(II) chloride dihydrate         CuCl2 * 2H2O         0.3 g
Boric acid                            H3BO3                0.6 g
Sodium molybdate dihydrate            Na2MoO4 * 2H2O       0.5 g
20 Zinc acetate dehydrate             Zn(CH3COO)2 * 2H2O   2.6 g
to 1 l deionized H2O
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Glycerol feed solution
2 l          of deionized water
25 211 g     of sodium sulfate
13.6 g       of iron(II) sulfate heptahydrate
8.8 kg       of 99.5% glycerol
220 ml       of trace element solution
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30 Rhamnose feed solution
703 g        of deionized water
297 g        of rhamnose monohydrate
```

The fermentation is carried out at a temperature of 37°C. The
35 gassing is regulated between 8-30 l/min, while the rotation speed
of the stirrer is regulated at 400-1500 l/min, in order to
maintain a pO_2 of not less than 20%. After a fermentation period
of 1 h, the culture is induced with IPTG (0.15 mM). 18.5 g of
rhamnose feed solution are then added. After the initially
40 introduced quantity of glycerol has been consumed, glycerol is
then fed in continuously. After a fermentation period of 44 h,
cell suspensions of 50 g DBM/l and 50 to 60 kU/l are obtained.
The cells are cooled down to 4°C.

45 Example 2: Activity test

50 µl of cell suspension are pipetted into 880 µl of sodium
potassium phosphate buffer (10 mM) and the whole is equilibrated
at 30°C. The reaction is started by adding 20 µl of methanolic
mandelonitrile solution (12%). After 10 min, the enzyme reaction
5 is stopped by adding 50 µl of 1M HC. The cell mass is centrifuged
off and the concentration of mandelic acid in the supernatant is
measured by means of HPLC (ODS Hypersil 100*2.0 mm, mobile phase:
75% H_3PO_4 (14.8 mM)/25% methanol; flow rate: 0.5 ml/min;
injection volume: 2 µl; column temperature: 40°C; detection:
10 210 nm; mandelic acid retention time: 0.9 min).

Example 3: Storing with benzaldehyde:

14 h after the end of the fermentation, the cell suspension was
15 adjusted to a pH of 6.0, 6.6 or 7.2 with NaOH or H_2SO_4 and then
treated with benzaldehyde. The samples were stored at 4°C or 22°C.
Enzyme activity was determined at 0.6, 3.6 and 6.6 days after the
end of the fermentation.

20 Storage at 22°C:

	pH	Storage period		
		0.6 d	3.6 d	6.6 d
		Activity in kU/l		
Without addition	7.2	51.0	49.0	48.7
1 mM benzaldehyde	7.2	55.8	50.4	48.7
5 mM benzaldehyde	7.2	47.1	51.7	52.9
10 mM benzaldeyde	7.2	53.8	52.7	51.3
Without addition	6.6	51.5	50.5	50.9
1 mM benzaldehyde	6.6	53.2	53.0	53.1
5 mM benzaldehyde	6.6	47.1	54.3	58.0
10 mM benzaldeyde	6.6	51.3	49.4	55.4
Without addition	6.0	54.8	45.6	44.5
1 mM benzaldehyde	6.0	55.1	50.6	51.0
5 mM benzaldehyde	6.0	51.8	51.5	54.9
10 mM benzaldeyde	6.0	51.3	53.0	49.2

Storage at 4°C:

	pH	Storage period		
		0.6 d	3.6 d	6.6 d
		Activity in kU/l		
Without addition	7.2	51.0	48.6	47.8
1 mM benzaldehyde	7.2	55.8	46.8	47.5
5 mM benzaldehyde	7.2	47.1	48.3	50.7
10 mM benzaldeyde	7.2	53.8	51.2	49.2
Without addition	6.6	51.5	49.5	45.3
1 mM benzaldehyde	6.6	53.2	52.3	52.5
5 mM benzaldehyde	6.6	47.1	52.0	55.7

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10 mM benzaldeyde	6.6	51.3	55.5	51.9
Without addition	6.0	54.8	42.8	34.9
1 mM benzaldehyde	6.0	55.1	49.9	48.3
5 mM benzaldehyde	6.0	51.8	52.3	53.0
10 mM benzaldeyde	6.0	51.3	51.5	50.2

Example 4: Storing with CBA:

14 h after the end of the fermentation, the cell suspension was adjusted to a pH of 6.0, 6.6. or 7.2 with NaOH or H_2SO_4 and then treated with 2-chlorobenzaldehyde. The samples were stored at 4°C or 22°C. The enzyme activity was determined at 0.6, 3.6 and 6.6 days after the end of the fermentation.

Storage at 22°C:

	pH	Storage period		
		0.6 d	3.6 d	6.6 d
		Activity in kU/l		
Without addition	7.2	51.0	49.0	48.7
1 mM 2-chlorobenzaldehyde	7.2	51.3	52.8	53.2
5 mM 2-chlorobenzaldehyde	7.2	53.3	51.4	50.1
10 mM 2-chlorobenzaldehyde	7.2	48.3	52.9	54.0
Without addition	6.6	51.5	50.5	50.9
1 mM 2-chlorobenzaldehyde	6.6	48.8	55.0	57.2
5 mM 2-chlorobenzaldehyde	6.6	50.6	56.7	55.5
10 mM 2-chlorobenzaldehyde	6.6	47.4	56.2	58.6
Without addition	6.0	54.8	45.6	44.5
1 mM 2-chlorobenzaldehyde	6.0	52.4	53.8	54.5
5 mM 2-chlorobenzaldehyde	6.0	52.5	55.0	59.1
10 mM 2-chlorobenzaldehyde	6.0	53.5	55.7	52.4

Storage at 4°C:

	pH	Storage period		
		0.6 d	3.6 d	6.6 d
		Activity in kU/l		
Without addition	7.2	51.0	48.6	47.8
1 mM 2-chlorobenzaldehyde	7.2	51.3	48.3	45.2
5 mM 2-chlorobenzaldehyde	7.2	53.3	51.2	48.1
10 mM 2-chlorobenzaldehyde	7.2	48.3	51.0	49.9
Without addition	6.6	51.5	49.5	45.3
1 mM 2-chlorobenzaldehyde	6.6	48.8	55.1	54.7
5 mM 2-chlorobenzaldehyde	6.6	50.6	56.3	53.6
10 mM 2-chlorobenzaldehyde	6.6	47.4	55.0	58.5
Without addition	6.0	54.8	42.8	34.9
1 mM 2-chlorobenzaldehyde	6.0	52.4	53.5	56.9
5 mM 2-chlorobenzaldehyde	6.0	52.5	55.6	53.8
10 mM 2-chlorobenzaldehyde	6.0	53.5	55.7	47.6

Example 5: Long-term storage:

The cell suspension was adjusted to pH 6.6 after which
5 2-chlorobenzaldehyde was added to a concentration of 1.35 mM and
the cell suspension was stored at 4°C. The course of the activity
is depicted in Fig. 2.

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